

CERTIFICATE OF TRANSLATION

I, SHUSAKU YAMAMOTO, patent attorney of Fifteenth Floor, Crystal Tower, 1-2-27 Shiromi, Chuo-ku, Osaka 540-6015, Japan HEREBY CERTIFY that I am acquainted with the English and Japanese languages and that the attached English translation is a true English translation of what it purports to be, a translation of Japanese Patent Application No. 8-187945 filed on 17 July, 1996 in the name of KANEGAFUCHI KAGAKU KOGYO KABUSHIKI KAISHA.

Additionally, I verify under penalty of perjury under the laws of the United States of America that the foregoing is true and correct.

Executed this 22th day of January, 2004.



SHUSAKU YAMAMOTO

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[Title of the Invention] DIAGNOSTIC DRUGS FOR AUTOIMMUNE
DISEASES AND INFLAMMATORY DIS-
EASES

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[Name of the Document] SPECIFICATION

[Title of the Invention] Diagnostic drugs for autoimmune diseases and inflammatory diseases

[Claims]

[Claim 1] A diagnostic drug for autoimmune diseases and/or inflammatory diseases, comprising at least one of a polypeptide selected from an HMG-1 family, a polypeptide selected from an HMG-2 family, a fragment thereof which is reactable with an antibody of autoimmune disease and/or inflammatory disease patients.

[Claim 2] A diagnostic drug according to claim 1, wherein the autoimmune disease is ulcerative colitis.

[Claim 3] A diagnostic drug for autoimmune diseases according to claim 2, wherein the polypeptide is selected from human, bovine, porcine, chicken, mouse or rat HMG-1 or HMG-2.

[Claim 4] A kit for diagnosing autoimmune diseases and/or inflammatory diseases, comprising at least one of a polypeptide selected from an HMG-1 family, a polypeptide selected from an HMG-2 family, a fragment thereof which is reactable with an antibody of autoimmune disease and/or inflammatory disease patients.

[Claim 5] A kit according to claim 4, wherein the autoimmune disease is ulcerative colitis.

[Claim 6] A kit for diagnosing for autoimmune diseases and/or inflammatory diseases according to claim 5, wherein the polypeptide is selected from human, bovine, porcine, chicken, mouse or rat HMG-1 or HMG-2.

[Claim 7] A method for detecting an antibody of autoimmune disease and/or inflammatory disease patients, comprising the step of reacting a reagent including at least one of a polypeptide selected from an HMG-1 family, a polypeptide selected from an HMG-2 family, a fragment thereof which is reactable with an antibody of autoimmune disease and/or inflammatory disease patients with a bodily fluid of autoimmune disease and/or inflammatory disease patients.

[Claim 8] A method according to claim 7, wherein the autoimmune disease is ulcerative colitis.

[Claim 9] A method according to claim 8, wherein the polypeptide is selected from human, bovine, porcine, chicken, mouse or rat HMG-1 or HMG-2.

[Detailed Description of the Invention]

[0001]

[Field of the Invention]

The present invention relates to a diagnostic drug for and a kit for diagnosing autoimmune diseases or inflammatory diseases, and a method for detecting an antibody of an autoimmune disease or inflammatory disease patient using HMG-1, HMG-2, or a fragment of a polypeptide thereof with which the antibody of the autoimmune disease patient or the inflammatory disease patient reacts. In particular, the present invention relates to a diagnostic drug for or a kit

for diagnosing ulcerative colitis, and a method for detecting an antibody of a patient of ulcerative colitis, using HMG-1, HMG-2, or a fragment of a polypeptide thereof with which the antibody of such a patient reacts.

[0002]

[Prior Art]

It has been reported that various anti-neutrophil cytoplasmic antibodies (ANCA) are involved in inflammatory diseases, especially inflammatory bowel diseases. ANCA are antibodies capable of being detected by indirect immunofluorescence assay (IIF) and are classified into cytoplasmic-ANCA (cANCA) and perinuclear-ANCA (pANCA). cANCA is detected in Wegener's granulomatosis patients at a high frequency, and the antigen to cANCA is proteinase-3 (hereinafter, referred to as "PR-3"). pANCA is detected in microscopic polyangiitis, and pauci-immune necrotizing crescentic glomerulonephritis (NCGN) patients at a high frequency, and the antigen was identified to be myeloperoxidase (MPO-ANCA). Subsequently, it was found that MPO-ANCA is lower in the disease specificity than PR-3 but is detected at a high frequency in pathological states exhibiting necrotizing angiitis, which is one of the angiitis syndromes of blood capillary and micro-blood capillary. Thus, ANCA plays an important role in early-phase diagnosis and differential diagnosis of antiititis syndromes. That is, early-phase diagnosis and differential diagnosis of angiitis syndromes are made possible by determining whether the patient is ANCA-positive or not and, when the patient is ANCA-positive, determining what the subset is.

[0003]

Recently, pANCA has been found in the patients suffering from inflammatory diseases including inflammatory bowel diseases (IBD) such as ulcerative colitis (UC), rheumatoid arthritis (RA), systemic lupus erythematosus (SLE), autoimmune hepatitis (AIH), malignant tumors, amebic abscesses, and sweet disease. As antigens to pANCA, lactoferrin, cathepsin G, elastase, lysozyme and the like have been identified. Causes of these diseases and relationship of these antigens and the diseases have been studied. However, the specificity of these antigens to pANCA is low, which suggests that there are other antigens involved.

[0004]

Generally, the ratios at which ANCA is detected in the patients (percentages of positive patients) of ulcerative colitis (UC) and Crohn's disease (CD), which are both inflammatory bowel diseases, by the indirect immunofluorescence assay (IIF) are 40 to 87% and 6 to 27%, respectively. Judging by the staining pattern, pANCA is detected at a high percentage of 80 to 95% in the case of ulcerative colitis, whereas pANCA and cANCA are equivalently detected in the case of Crohn's disease (CD).

[0005]

As antigens to ANCA detected in ulcerative colitis (UC) and Crohn's disease (CD), various antigens have been reported including lactoferrin, cathepsin G, myeloperoxidase, and myeloperoxidase+elastase, and myeloperoxidase+elastase+cathepsin G. However, antigens specific to these diseases have not been identified so far.

[0006]

Standard diagnostic methods for ulcerative colitis (UC) include endoscopy, biopsy, and X-ray examination. These methods are costly, painful, and time-consuming. Indirect immunofluorescence (IIF) assay has been used as a serodiagnosis for ulcerative colitis. However, this method is not sufficiently sensitive and tends to have an increased background signal. Serodiagnosis, by which neutrophils and other cells are fixed on a plate with ethanol, has a further disadvantage in that the result depends on the state of the cells and the fixing technique and is not sufficiently reliable. As described above, a specific and simple diagnostic method for ulcerative colitis (UC) has not been developed.

[0007]

[Problems to be Solved by the Invention]

As can be appreciated, it leads to establishment of easier diagnosis of an autoimmune disease and appropriate therapeutic strategies to identify an antigen of an autoimmune disease patient. Especially, it leads to establishment of easier diagnosis of ulcerative colitis (UC) and appropriate therapeutic strategies to identify an antigen to anti-neutrophil cytoplasmic antibodies (ANCA) in an ulcerative colitis (UC) patient. It is demanded to isolate an antigen specific to autoimmune diseases and inflammatory diseases, especially ulcerative colitis and to develop a simple method for detecting an antibody using the antigen.

[0008]

[Means for Solving the Problems]

As a result of active studies in order to overcome the above-described problems with the prior art, the present

inventors succeeded in isolating high mobility group protein-1 (HMG-1) and high mobility group protein-2 (HMG-2), which are known proteins, as a novel antigen to which an antibody of autoimmune disease and inflammatory disease patients, especially ulcerative colitis patients. The present inventors found that detection of the antibody against the antigen can be a marker of ulcerative colitis, and thus completed the present invention.

[0009]

The present invention relates to a diagnostic drug for autoimmune diseases and/or inflammatory diseases, comprising at least one of a polypeptide selected from an HMG-1 family, a polypeptide selected from an HMG-2 family, a fragment thereof which is reactable with an antibody of autoimmune disease and/or inflammatory disease patients, a kit for diagnosing these diseases, and a method for detecting an antibody of autoimmune disease and/or inflammatory disease patients.

[0010]

In a preferred embodiment of the invention, the autoimmune disease is ulcerative colitis.

[0011]

In a preferred embodiment of the invention, the polypeptide is selected from human, bovine, porcine, chicken, mouse or rat HMG-1 or HMG-2.

[0012]

Thus, the objectives of the present invention are achieved.

[0013]

[Embodiments of the Invention]

The polypeptide used for the present invention is a polypeptide selected from the HMG-1 family or HMG-2 family or a fragment thereof.

[0014]

The HMG-1 (high mobility group protein-1) family refers to polypeptides having an amino acid homology of 90% or more with human HMG-1 indicated by SEQ NO: 1 and includes, for example, bovine HMG-1 (SEQ NO: 3), porcine HMG-1 (SEQ NO: 4), and rat HMG-1 (SEQ NO: 5). Human HMG-1 is preferable, but commercially available bovine HMG-1 is also usable. Figure 9 compares the amino acid sequences of these types of HMG-1.

[0015]

The HMG-2 (high mobility group protein-2) family refers to polypeptides having an amino acid homology of 80% or more with human HMG-2 indicated by SEQ NO: 2 and includes, for example, porcine HMG-2 (SEQ NO: 6), partial sequence of bovine HMG-2 (SEQ NO: 7), chicken HMG-2 (SEQ NO: 8), chicken HMG-2a (SEQ NO: 9), and mouse HMG-2 (SEQ NO: 10). Human, HMG-2 is preferable. Commercially available bovine HMG-2 is also usable. Figure 9 compares the amino acid sequences of these types of HMG-2.

[0016]

The polypeptides belonging to the HMG-1 or HMG-2 family include a polypeptide having deletion, substitution or addition of one or more amino acid, or a fragment thereof

which can react with an antibody from autoimmune disease and/or inflammatory disease patients.

[0017]

The fragment refers to a fragment which can react with an antibody from autoimmune disease and/or inflammatory disease patients among the fragments of polypeptides belonging to the HMG-1 or HMG-2 family. The fragment can be prepared using an appropriate proteolytic enzyme. Whether the prepared fragment reacts with an antibody or not can be determined by reacting the fragment with serum obtained from autoimmune disease or inflammatory disease patients. This method is well known among those skilled in the art, and the same technique as used for antibody detection described below can be used.

[0018]

HMG-1 and HMG-2 are proteins ubiquitously contained in all the cells and thus can be prepared by extraction from any organ, tissue, or cell. HMG-1 and HMG-2 are extracted from, for example, human thymus, porcine thymus, bovine thymus, human placenta, neutrophil, and HL-60 cell line.

[0019]

A polypeptide belonging to the HMG-1 or HMG-2 family is produced by culturing the above-mentioned cells producing the polypeptide, or by introducing a vector incorporating a gene encoding the polypeptide into a host cell and expressing the polypeptide. A polypeptide having deletion, substitution or addition of one or more amino acid can be produced by a well-known method based on, for example, a HMG-1 or HMG-2 gene sequence; for example, by modifying the gene

sequence through site-directed mutagenesis or deletion mutagenesis using M13 phage and expressing the polypeptide. The host cell can be a prokaryote or eukaryote; e.g., bacteria such as Escherichia coli and Bacillus, yeast, mold, insect cell, and mammal cell. Polypeptide can be purified by a well-known method, for example, chromatography such as gel filtration chromatography, ion exchange chromatography, affinity chromatography, or reverse phase liquid chromatography performed independently or in combination. Preferably, reverse phase HPLC or ion exchange chromatography is usable. A column for reverse phase HPLC can be any of various commercially available columns, but a protein separation column, for example, YMC-protein RP column (YMC) is preferably usable.

[0020]

The autoimmune diseases to which the present invention relates include ulcerative colitis. Ulcerative colitis in the present invention has the following symptoms. Ulcerative colitis refers to an idiopathic, nonspecific inflammatory disease of large intestine, especially rectum, which mainly attacks mucosa and submucous layer. This disease often attacks adults of 30 years old or younger, but is also found in children and adults of 50 years old or older. The cause is not known and is considered to be related to immunological mechanisms, or genetic or psychological factors. Usually, diarrhea with hematochezia and various systemic symptoms are shown. When it lasts long and attacks the entire large intestine, it tends to be malignant. Refractory ulcerative colitis refers to cases in which the ulcerative colitis is under precise medical therapy and still (1) shows chronic persistence, (2) is active for six months

or longer after recrudescence or (3) repeats recrudescence frequently.

[0021]

An antibody is a component which exists in a bodily fluid of an autoimmune disease or inflammatory disease patient and is induced by a specific antigenic substance. Specifically, an antibody of a refractory ulcerative colitis patient or an antibody of an ulcerative colitis patient respectively refer to a component contained in the bodily fluid such as serum of a patient diagnosed to have refractory ulcerative colitis or ulcerative colitis, respectively. Antibodies of ulcerative colitis patients include, for example, IgM, IgG, IgE, IgD and IgA.

[0022]

A diagnostic drug according to the present invention includes a polypeptide contained in the HMG-1 or HMG-2 family or a fragment thereof. The diagnostic drug includes at least one type of polypeptide contained in the HMG-1 or HMG-2 family or a fragment thereof. A mixture of HMG-1 and HMG-2 is preferably used.

[0023]

This diagnostic drug reacts with an antibody of an autoimmune disease patient to form an antigen/antibody complex. Accordingly, the drug can include a further component for detecting the resultant antigen/antibody complex. Such a component is suitable to methods such as, for example, precipitation reaction method, ELISA assay, RIA, and Western blotting.

[0024]

The polypeptide contained in the HMG-1 or HMG-2 family or a fragment thereof is used in a diagnostic kit. The diagnostic kit can include, for example, an ELISA plate having a polypeptide contained in the HMG-1 or HMG-2 family or a fragment thereof immobilized thereon and a reagent for detecting an antigen/antibody complex bonded to an antibody of an autoimmune disease patient. The reagent includes a component suitable to methods such as precipitation reaction, ELISA, RIA, and Western blotting. As a detecting reagent, a secondary antibody reagent is, for example, used for an ELISA assay. The secondary antibody reagent can be generally tagged with a tagging agent used in immune measurement methods. Usable tagging agents include, for example, radioisotope (e.g., ^{32}P , ^3H , ^{125}I), enzyme (e.g., β -galactosidase, peroxidase, alkali phosphatase, glucoseoxidase, lactate oxidase, alcoholoxidase, monoamineoxidase), coenzyme and prosthetic group (e.g., FAD, FMN, ATP, biotin, heme), fluorescein derivatives (e.g., fluorescein isothiocyanate, fluorescein thioflubamyl), rhodamine derivatives (e.g., tetramethyl rhodamine B isothiocyanate), Umbelliferone and 1-anilino-8-naphthalenesulfonic acid, and luminol derivatives (e.g., luminol, isoluminol).

[0025]

The kit can be of a format in which the antigen is accommodated in an appropriate carrier in the format of container, resin, membrane, film or the like is fixed on an appropriate carrier in the format of container, resin, membrane, film or the like. The carrier and the antigen can be bonded to each other by a known method such as, for example, physisorption, ionic bond, covalent bond, or entrapping.

Physisorption is especially preferable due to the simplicity. The antigen and the carrier can be bonded to each other directly or through another substance (e.g., spacer) interposed therebetween. The immobilized antigen can be blocked by a blocker such as gelatin or BSA to restrict non-specific bonding.

[0026]

A method for detecting an antibody of an autoimmune disease or ulcerative colitis patient according to the present invention includes the step of reacting a polypeptide selected from the HMG-1 family, a polypeptide selected from the HMG-2 family, or a fragment thereof (antigen) with a bodily fluid component of an autoimmune disease or inflammatory disease patient. The reaction conditions can be well known conditions in the art. A method for detecting a substance obtained by the antigen/antibody reaction can also be a known method in the art. Usable detection methods include, for example, precipitation reaction, ELISA, RIA and Western blotting. Detection is performed as follows, for example. Serum from the patient appropriately diluted and the antigen are reacted with each other and washed. Next, an alkali phosphatase-tagged anti-human IgG antibody as a secondary antibody is added and reacted. Then, p-nitrophenyl phosphoric acid, which is a substrate of alkali phosphatase, is added and colored. The absorbance at 405 nm is measured. Thus, measurement for an anti-HMG-1 antibody and an anti-HMG-2 antibody can be performed.

[0027]

Hereinafter, using an ulcerative colitis patient as an example, a method of screening an antigen capable of

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reacting with an antibody of the patient and specifying that the antigen is HMG-1 and HMG-2.

[0028]

First, blood is sampled from an ulcerative colitis patient to obtain a serum component. Next, the serum component is measured for anti-neutrophil cytoplasmic antibody (ANCA) by indirect immunofluorescence assay. From peripheral blood of healthy persons, a neutrophil fraction is separated by specific gravity centrifugation. Next, the neutrophil fraction is treated to obtain neutrophil lysate and Western blotting is performed. For example, 10^6 neutrophils are dissolved in a sample buffer containing 2-mercaptoethanol and SDS and boiled for 10 minutes. After the resultant substance is rapidly cooled with ice, SDS-polyacrylamide gel electrophoresis is performed. After electrophoresis, the protein band is transferred to a nylon membrane and non-specific bonding is blocked using skim milk or the like.

[0029]

Serum from an ANCA-positive patient is applied to protein A column to purify an IgG antibody fraction. The purified IgG antibody fraction and the neutrophil lysate transferred to the nylon membrane are reacted to each other. After the nylon membrane is washed, chemical light emission is caused by, for example, a detecting agent such as ECL kit (Amersham), thereby detecting the band. Thus, the antigen is determined to exist. Alternateness with other diseases, for example, Crohn's disease is examined and the specificity is determined.

[0030]

According to the above-described method, antigenic polypeptide can be confirmed to exist. Such an antigenic polypeptide can be purified by any known protein purification method.

[0031]

Alternatively, a cell line producing an antigenic polypeptide can be specified using the above-described antibody. An antigenic polypeptide can be produced using such a cell line and purified by any known protein purification method.

[0032]

By the above-described method, the antigen of 28kDa to the anti-neutrophil cytoplasmic antibody could be specified. As described in an example below, an antigen of 28/39.5/44/47/58kDa was detected in ten out of 24 ANCA-positive patients. Five out of the seven patients had been diagnosed to have refractory ulcerative colitis. No positive band was detected in the ulcerative colitis patients who were found to be ANCA-negative by indirect immunofluorescence assay. The molecular weight of the antigen according to the present invention is determined by 10%SDS-polyacrylamide gel electrophoresis.

[0033]

As a result of screening cells producing a 28kDa antigen by the above-described screening method, HL-60 cell line (ATCC CCL-240), which is a neutrophil cell derived from premyelogenetic leukemia, was found to have a 28kDa antigen. It was also found that the HL-60 cell line includes 29kDa

antigen as well as 28kDa antigen. From the HL-60 cell line, the 28kDa and 29kDa antigens can be purified. The 28kDa and 29kDa antigens can be purified by any known protein purification method. For example, HL-60 cell line is cultured in a RPMI1640 medium containing 5% FCS added thereto, and the cells are dissolved in 6M guanidine hydrochloride and treated with sonication to inactivate the proteolytic enzymes. The protein is completely dissolved and concentrated by dialyses, for example, ultrafiltration, and simultaneously the solution is substituted with PBS. From the resultant aqueous solution, a 28kDa antigen and a 29kDa antigen can be purified. Preferably, reverse phase HPLC is usable. By performing fractionation with reverse phase HPLC using acetonitrile concentration gradient, a 28kDa antigen and a 29kDa antigen having a purity of 90% or more can be purified. The conditions for reverse phase HPLC using acetonitrile concentration gradient can be well known conditions.

[0034]

As a result of analyzing the amino acid sequence of the purified proteins, the 29kDa was identified as high mobility group protein-1 (HMG-1) and the 28kDa was identified as high mobility group protein-2 (HMG-2).

[0035]

The 29kDa antigen and the 28kDa antigen reacted with an antibody of an ulcerative colitis patient in a dose dependent manner. Commercially available bovine HMG-1 and HMG-2 (Wako Pure Chemical) reacted with an antibody of an ulcerative colitis patient. Accordingly, HMG-1 and HMG-2 families are considered to be antigens of ulcerative colitis.

[0036]

The specificity to the disease can be detected by checking the response between peripheral blood lymphocyte and HMG-1 and HMG-2 of a patient considered to have ulcerative colitis. The disease can be detected by measuring whether or not T lymphocyte proliferates in response to HMG-1, HMG-2 or a synthetic peptide thereof which is immuno-responsive.

[0037]

HMG-1 and HMG-2 are identified as antigens to pANCA. HMG-1 and HMG-2 were previously identified as intranuclear proteins. Accordingly, antibodies thereto can be possibly detected in p-ANCA-positive patients who do not have ulcerative colitis. Since the ELISA assay is more sensitive than indirect immunofluorescence assay, it is possible to detect an anti-HMG-1 antibody and an anti-HMG-2 antibody in the diseases which have been considered to be ANCA-negative or to have a low ANCA-positive percentage. These diseases includes, for example, autoimmune diseases including ANCA-positive diseases and inflammatory diseases, e.g., ulcerative colitis, Crohn's disease, chronic rheumatoid arthritis, systemic lupus erythematosus, autoimmune hepatitis, hepatitis B, hepatitis C, Wegener's granulomatosis, leukocyte destructive angiitis, crescentic glomerulonephritis, pauci-immune necrotizing crescentic glomerulonephritis (rapidly progressing nephritis), microscopic polyangiitis, Churg-Strauss syndrome, primary biliary cirrhosis, primary sclerogenic cholangitis, Behçet's disease, polymyositis, dermatomyositis, Sjögren's syndrome, progressive systemic sclerosis (scleroderma), mixed and connective-tissue disease.

[0038]

Hereinafter, the present invention will be described by way of illustrative examples with reference to the accompanying drawings.

[0039]

[Examples]

(Example 1) Detection of anti-neutrophil cytoplasmic antibody (ANCA) by the indirect immunofluorescence assay (IIF)

Peripheral blood was sampled from 35 ulcerative colitis patients (16 males and 19 females) and centrifuged (4°C, 13 minutes, 2000 rpm), thereby obtaining serum components. The percentage of the positive components with respect to the anti-neutrophil cytoplasmic antibody (ANCA) was measured by the indirect immunofluorescence assay. As a control, blood sampled from 10 Crohn's disease patients (9 males and 1 female) was treated in a similar manner and subjected to the same measurement.

[0040]

The measurement conditions for the indirect immunofluorescence assay using ethanol-fixed human neutrophils will be described below.

[0041]

First, peripheral blood is treated with specific gravity centrifugation using the Ficoll-Hypaque technique to separate the neutrophils, and the neutrophils are applied to slides at the ratio of 10^5 neutrophils/slide by cytospin. Then, the slides are dried by cool air from a dryer and washed

with PBS (0.8% NaCl/0.02% KC1/10mM Na₂HPO₄/1.5mM KH₂PO₄ pH7.4). Sample sera are each diluted at 1:10 with PBS, and 20 μ l of the resultant sample is put on the slides and reacted at room temperature for 1 hour in a humidified chamber. After the reaction is completed, the resultant substance is washed with PBS. FITC-tagged rabbit anti-human IgG F(ab')₂ antibody is diluted at 1:20 with PBS, and 20 μ l of the resultant antibody is put on the slide and reacted at room temperature for 30 minutes in a humidified chamber. After the reaction is completed, the resultant substance is washed with PBS. The washed substance is embedded with glycerol diluted to 1:9 with PBS and observed with a fluorescent microscope. The observation results obtained in this manner are shown in Table 1. Among the 35 ulcerative colitis patients, 24 patients had ANCA (the percentage of the positive components: 69%; see Table 1).

Regarding the stain pattern obtained by the indirect immunofluorescence (IIF) assay, the anti-neutrophil cytoplasmic antibodies (ANCA) of the 24 positive patients mostly had pANCA (pANCA for 22 patients; nuclear-ANCA for 2 patients; see Table 1). The two patients exhibiting nuclear-ANCA are patient Nos. 24 and 25 in Table 3 shown below.

[0042]

[Table 1]

ANCA detected in ulcerative colitis and Crohn's disease patients by IIF

| Patient | Number of patients | Percentage of positive patients(%) | Staining pattern | | | Titer |
|--------------------|--------------------|------------------------------------|------------------|-------------|---------|------------|
| | | | Perinuclear | Cytoplasmic | Nuclear | |
| Ulcerative colitis | 35 | 24(59) | 22 | 0 | 2 | 1/10-1/320 |
| Crohn's disease | 10 | 6(50) | 2 | 4 | 0 | 1/10-1/40 |
| Healthy | 39 | 0(0) | | | | |

[0043]

(Example 2) Study on known antigens to ANCA

Antigens to ANCA were studied for the 35 ulcerative colitis patients.

[0044]

Five micrograms/ml of myeloperoxidase (MPO), 5 µg/ml of cathepsin G (CaG), and 10 µg/ml of lactoferrin (LF) were prepared, injected into 96-well microtiter plates in the quantities of 50 µl/well, 50 µl/well and 100 µl/well, respectively, and stored at 4°C overnight to coat the wells. After the coating, the solution was removed, and 5% BSA (bovine fetus serum)-containing PBS (5% BSA/PBS) was added to each well and reacted for 30 minutes. Then, 5% BSA/PBS was removed. The serum obtained from the patients was diluted 10-fold using 5% BSA/PBS, added to the microtiter plates, and reacted at room temperature for 24 hours. The reaction liquid was removed, and each well was washed 5 times with 1% BSA/PBS/0.5% Tween20. After the washing, alkali phosphatase (ALP)-tagged ovine anti-human IgG antibody was diluted 1000-fold with 5%

BSA/PBS, added to each well and reacted at room temperature for 24 hours. After the reaction was completed, each well was washed 5 times with 1% BSA/0.5% Tween20/PBS. After the washing, 100 μ l of 10% solution of p-nitrophenyl phosphate (final concentration: 5 mg/ml) in diethanolamine (diethanolamine 50 ml+distilled water 450 ml) was added and color-developed at room temperature for 30 minutes. After the color development, the absorbance at 405 nm was measured.

[0045]

According to this method, the anti-myeloperoxidase antibody was not detected in any of the samples. Among the 24 patients who were found to be positive by IIF, 9 patients were positive regarding the anti-cathepsin antibody and 3 patients were positive regarding the anti-lactoferrin antibody. For the other 12 patients, the antigen responsible for the positive reaction was not specified (see Tables 2 and 3 below).

[0046]

(Example 3) Screening of antigen to anti-neutrophil cytoplasmic antibody (ANCA)

Regarding the 24 ANCA-positive patients, Western blotting was performed using the neutrophil lysate.

[0047]

Peripheral blood was sampled from the patients, and neutrophil fractions were prepared by the centrifugation using the Ficoll-Hypaque technique. 10^6 neutrophils were suspended in 8 μ l of PBS per well. Then, 2 μ l of sample buffer containing 25%2-mercaptoethanol and 10% SDS (0.2M

Tris-HCl pH6.8/10% SDS/25% 2-mercaptoethanol/25% glycerol/0.01% BPB) was added and immediately boiled for 10 minutes, thereby obtaining an antigen solution. Electrophoresis was performed using SDS-polyacrylamide gel (SDS-PAGE). After the electrophoresis, the resultant substance was transferred to a Nylon membrane in a usual manner and reacted for 2 hours after skim milk was added in order to block non-specific bonding. Serum obtained from the patients in an amount of 320 μ l was applied to ProCep A (10 ml bed volume). Then, IgG fractions were eluted using 0.1M-glycine (pH3.0) and purified, thereby obtaining a 20 mg/ml solution of IgG. The above-prepared Nylon membrane and 1 ml of the IgG solution diluted 8-fold with 5% skim milk were reacted at 4°C overnight. After being washed, the resultant substance was further reacted with 13 μ g/ml of peroxidase-bound anti-human IgG antibody. The resultant substance was chemically emitted with an ECL kit (Amersham), and the band was detected. The results are shown in Tables 2 and 3.

[0048]

[Table 2]

| Patient No. | IIF | MPO | CaG | LF | 28kDa antigen, etc. | Refractory type |
|-------------|-----|-----|-----|----|------------------------|--------------------|
| 1 | + | - | - | - | + | + |
| 2 | + | - | - | + | - | - |
| 3 | + | - | - | - | - | - |
| 4 | + | - | + | - | + | + |
| 5 | + | - | - | - | - | - |
| 6 | + | - | + | - | + | + |
| 7 | + | - | - | + | - | - |
| 8 | + | - | + | - | + (39.5KDa) | + |
| 9 | + | - | - | - | - | - |
| 10 | + | - | - | - | - | - |
| 11 | + | - | - | - | + (47KDa) | - |
| 12 | + | - | - | - | - | - |
| 13 | + | - | - | - | - | - |
| 14 | + | - | + | - | + | - |
| 15 | + | - | - | - | + (44KDa) | - |
| 16 | + | - | - | - | - | - |
| 17 | + | - | + | - | + | + |
| 18 | + | - | - | - | - | - |
| 19 | + | - | - | - | - | - |
| 20 | + | - | + | - | + | - |

In the table, "+" indicates positive reactivity or refractory type.

[0049]

[Table 3]

| Patient No. | IIF | MPO | CaG | LF | 28kDa antigen, etc. | Refractory type |
|-------------|-----|-----|-----|----|------------------------|--------------------|
| 21 | + | - | + | - | ÷ | + |
| 22 | + | - | - | + | - | ÷ |
| 23 | - | - | + | - | - | - |
| 24 | + | - | + | - | + (50KDa) | - |
| 25 | ÷ | - | - | - | - | - |
| 26 | - | - | - | - | - | - |
| 27 | - | - | - | - | - | - |
| 28 | - | - | - | - | - | - |
| 29 | - | - | - | - | - | - |
| 30 | - | - | - | - | - | - |
| 31 | - | - | - | - | - | - |
| 32 | - | - | - | - | - | - |
| 33 | - | - | - | - | - | - |
| 34 | - | - | - | - | - | - |
| 35 | - | - | - | - | - | - |

In the table, "+" indicates positive reactivity or refractory type.

[0050]

As a result, the sera of 11 patients among the 24 patients who were positive regarding the anti-neutrophil cytoplasmic antibody (ANCA) were found to contain an antigen capable of binding with either one of the 28/39.5/44/47/50/58kDa bands. Especially, the sera of seven patients among the 11 patients were confirmed to contain a substance capable of binding with the 28kDa band. Five out of seven patients containing the 28kDa band were diagnosed to have refractory ulcerative colitis (see Figure 1).

[0051]

In the sera obtained from the ulcerative colitis patients in which ANCA were not indicated by the indirect immunofluorescence assay (IIF), no antibody capable of binding with 28kDa was found. In the sera of the Crohn's disease patients used as the control, no antibody capable of binding with 28kDa was found.

[0052]

These results are summarized as follows. Among the 35 ulcerative colitis patients, 24 patients were ANCA-positive in accordance with IIF, and 11 patients were found to have an antigen capable of binding with ANCA by Western blotting. (All the 11 patients were ANCA-positive in accordance with IIF.) Among the 35 patients, 7 patients had refractory ulcerative colitis. Six out of the 7 patients were found to have an antigen capable of binding with ANCA by Western blotting, and the 28kDa band was found in 5 out of the 6 patients. No positive band was found in the serum of the Crohn's patients used as the control (see Figure 1).

[0053]

These experiment results suggest that expression of an antibody against the neutrophil 28kDa antigen can be a marker for predicting the seriousness (refractory type) of the ulcerative colitis and demonstrates the importance of isolation analysis of the antigen and the importance of developing a detection system for a simple antibody using an isolated antigen.

[0054]

(Example 4) Confirmation of existence of 28kDa and 29kDa antigens in HL-60 cells

HL-60 cell lines, which are neutrophil-type cells derived from premyelogenetic leukemia, were cultured in a RPMI1640 medium containing 5% FCS added thereto, and neutrophil lysate was created in the same method as in Example 3. Using the lysate, Western blotting was performed in the same manner as in Example 3. The results are shown in Figure 2 together with the results obtained from the positive control using the neutrophil lysate. Based on the results, the 28kDa antigen and the 29kDa antigen capable with binding with the antibody fraction obtained from the ulcerative colitis patients were confirmed to exist.

[0055]

(Example 5) Purification of 28kDa and 29kDa antigens from HL-60 cells

The HL-60 cells which were confirmed to contain the antigens in Example 4 were cultured in a RPMI1640 medium containing 5% FCS added thereto. When 1×10^5 /ml cells were

increased to 2×10^6 /m, the cells were centrifuged and recovered. 2×10^8 cells were dissolved by addition of ten milliliters of 6M guanidine hydrochloride. Sonic treatment (USP600, Shimadzu) was performed for 10 minutes. Such treatment completely dissolved the cells. After distilled water in the same amount as the resultant solution was added, centrifugation was performed for 30 minutes at 80,000xg and supernatant was recovered. The supernatant was placed in an Amicon ultrafiltration device (Amicon) equipped with YM3 (Amicon), which is a membrane having a molecular cut of 3,000. Filtration was performed while PBS was added, thereby finally obtaining 4 ml of PBS solution. The PBS solution was centrifuged for 30 minutes at 80,000xg to remove the precipitate, thereby recovering supernatant. The recovered supernatant was used as the antigen-containing sample, and 28kDa and 29kDa fractions were separated from the sample using HPLC. Using a YMC-pack protein RP column (YMC), proteins were eluted from acetonitrile having a concentration of 16% by 48% concentration gradient. The results are shown in Figure 3. No. 9 peak fraction in Figure 3 was collected and lyophilized. The lyophilize sample was re-dissolved in PBS. Using the YMC-pack protein RP column (YMC) again, proteins were eluted from acetonitrile having a concentration of 24% by 36% concentration gradient. As the HPLC system, the LC-7A system (Shimadzu) was used. The results are shown in Figure 4. No. 5 and No 6 peak fractions were collected and lyophilized. The lyophilizate was electrophoresed on SDS-PAGE, and transferred to a PVDF membrane (Amersham) by Western blotting. After staining with Ponceau S, the 28kDa and 29kDa bands were cut out and recovered. The results are shown in Figure 5. In the SDS-PAGE in the purified antigens, the two types of proteins were separated from each other and were

identifiable.

[0056]

(Example 6) Partial amino acid sequencing and homology analysis

The membrane containing the 28kDa and 29kDa recovered in Example 5 was dried and then used for amino acid sequencing. The amino acid sequencing was performed using PPSQ-10, which is an automatic protein sequencer produced by Shimadzu. As a result, 32 amino acids from the N-terminal were sequenced for the 28kDa band. The sequence was as follows.

[0057]

Gly Lys Gly Asp Pro Asn Lys Pro Arg Gly Lys Met Ser Ser Tyr Ala Phe Phe
Val Gln Thr Xaa Arg Glu Glu His Lys Lys Lys His Pro Asp (SEQ ID NO 1 1)

As a result of a similar analysis of amino acid sequences of the 29kDa band, 32 amino acids from the N-terminal were sequenced. The sequence was as follows.

[0058]

Gly Lys Gly Asp Pro Lys Lys Pro Arg Gly Lys Met Ser Ser Tyr Ala Phe Phe
Val Gln Thr Xaa Arg Glu Glu His Lys Lys Lys His Pro Asp (SEQ ID NO 1 2)

(Example 7) Homology analysis of partial amino acid sequence

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Homology analysis of the amino acid sequences obtained in Example 6 was performed. Using the BLAST program of Altschul, S.F. et al. (J. Mol. Biol. vol. 205, 403-410), a homology search was performed for all the amino acid sequences included in the known data PIR. As a result, 31 out of 32 amino acids of the 29kDa antigen matched with those of non-histone nucleic protein HMG-1 (Reeck, G.R., Nucleic Acids Res. 17, 1989, vol. 17: 1197-1214), and 31 of 32 amino acids of the 28kDa antigen matched with those of HMG-2 (Majumdar, A. et al., Nucleic Acids Res. 1991, vol. 19: 6643). The 22nd cystine could not be identified in both of the antigens. Since cystine is not detected unless modifying a thiol group, the 22nd amino acid is considered to be cystine. In consideration of this assumption and the molecular weight measured by SDS-PAGE, the 28kDa and 29kDa antigens were identified as HMG-2 and HMG-1, respectively.

[0059]

(Example 8) Detection of anti-HMG-1 antibody and anti-HMG-2 antibody in UC patient serum by Western blotting

Western blotting was performed using a bovine HMG-1 and HMG-2 mixture (available from Wako Pure Chemical) as an antigen. A mixture of bovine HMG-1 (0.5 µg) and HMG-2 (0.5 µg) was dissolved in a sample buffer (Example 3), and heat-treated in a usual manner, thereafter SDS-PAGE was performed. After the SDS-PAGE was completed, the antigens were transferred to a PVDF membrane and reacted with the UC patient serum and with HRP-tagged anti-human IgG antibody in this order. Detection was performed by ECL kit. The results are shown in Figure 6. These results demonstrate that anti-HMG-1 antibody and anti-HMG-2 antibody exist in the UC

patient serum.

[0060]

(Example 9) Measurement of anti-HMG-1 antibody and anti-HMG-2 antibody by an ELISA assay

In each well of a 96-well ELISA plate (Nunc), 50 µm bovine HMG-1 or HMG-2 was added in a stepped manner by 50 µl at a time and left in a stationary state at 4°C for 24 to 36 hours. After excess antigen was removed, 200 µl of 5% BSA was added and left in a stationary state for 30 minutes or longer for performing blocking. Serum diluted 40-fold or 80-fold with 5% BSA from the patients added in an amount of 50 µl and left in a stationary state at room temperature for 2 hours. After the resultant substance was washed 5 times with 0.05% Tween/0.02% azide/PBS (washing liquid), 100 µl of alkali phosphatase-tagged goat anti-human IgG ($F(ab')_2$) (available from Funakoshi) diluted 1000-fold was added and reacted at room temperature for 2 hours. After each well was washed 5 times with the washing liquid, 100 µl of 0.1% p-nitrophenyl phosphate, disodium (pNPP) (Sigma) solution (10% diethanolamine solution) was added and reacted at room temperature for 20 to 25 minutes. The absorbance at 405 nm was measured.

[0061]

Figure 7 shows calibration curves of the positive controls. Based on these calibration curves, it was found that measurement for anti-HMG-1 antibody and/or anti-HMG-2 antibody can be performed by an ELISA assay using bovine HMG-1 and HMG-2. As a result of measuring the serum of 68 ulcerative colitis patients, 20 (29.4%) was positive, which

was significantly higher than that of healthy people (7.1%, 4/20 people). The value of mean+3SD or more of the healthy people as the control were considered positive. Among the ulcerative colitis patients, 5 out of 7 refractory ulcerative colitis patients were positive, which was high (71.4%).

[0062]

[Effect of the Invention]

HMG-1 and HMG-2 are identified as antigens to anti-neutrophil cytoplasmic antibodies (ANCA) in ulcerative colitis. Thus, simple detection of antibodies using the antigens are now possible.

Since HMG-1 and HMG-2 are identified as antigens to ANCA, antibodies thereto can be possibly detected in ANCA-positive patients who do not have ulcerative colitis. Since the ELISA assay is more sensitive than indirect immunofluorescence assay, it is possible to detect an anti-HMG-1 antibody and an anti-HMG-2 antibody in the diseases which have been considered to be ANCA-negative or to have a low ANCA-positive percentage. These diseases includes, for example, autoimmune diseases and inflammatory diseases. In addition to ulcerative colitis, these diseases specifically include Crohn's disease, chronic rheumatoid arthritis, systemic lupus erythematosus, autoimmune hepatitis, hepatitis B, hepatitis C, Wegener's granulomatosis, leukocyte destructive angiitis, crescentic glomerulonephritis, pauci-immune necrotizing crescentic glomerulonephritis (rapidly progressing nephritis), microscopic polyangiitis, Churg-Strauss syndrome, primary biliary cirrhosis, primary sclerogenic cholangitis, Behcet's disease, polymyositis, dermatomyositis, Sjögren's syndrome,

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progressive systemic scleroma (scleroderma), and mixed and connective-tissue disease.

[0063]

[Sequence listing]

SEQ ID NO : 1

LENGTH : 214

TYPE : amino acid

MOLECULAR TYPE : peptide

FEATURE : HMG-1

ORIGINAL SOURCE : Human

SEQUENCE

Gly Lys Gly Asp Pro Lys Lys Pro Arg Gly Lys Met Ser Ser Tyr Ala Phe Phe

5 10 15

Val Gln Thr Cys Arg Glu Glu His Lys Lys Lys His Pro Asp Ala Ser Val Asn

20 25 30 35

Phe Ser Glu Phe Ser Lys Lys Cys Ser Glu Arg Trp Lys Thr Met Ser Ala Lys

40 45 50

Glu Lys Gly Lys Phe Glu Asp Met Ala Lys Ala Asp Lys Ala Arg Tyr Glu Arg

55 60 65 70

Glu Met Lys Thr Tyr Ile Pro Pro Lys Gly Glu Thr Lys Lys Phe Lys Asp

75 80 85 90

Pro Asn Ala Pro Lys Arg Pro Pro Ser Ala Phe Phe Leu Phe Cys Ser Glu Tyr

95 100 105

Arg Pro Lys Ile Lys Gly Glu His Pro Gly Leu Ser Ile Gly Asp Val Ala Lys

110 115 120 125

Lys Leu Gly Glu Met Trp Asn Asn Thr Ala Ala Asp Asp Lys Gln Pro Tyr Glu

130 135 140

Lys Lys Ala Ala Lys Leu Lys Glu Lys Tyr Glu Lys Asp Ile Ala Ala Tyr Arg

145 150 155 160

Ala Lys Gly Lys Pro Asp Ala Ala Lys Lys Gly Val Val Lys Ala Glu Lys Ser

165 170 175 180

Lys Lys Lys Lys Glu Glu Glu Glu Asp Glu Glu Asp Glu Glu Asp Glu Glu Glu

185 190 195

Glu Glu Asp Glu Glu Asp Glu Glu Glu Asp Asp Asp Asp Glu

200 205 210

[0064]

SEQ ID NO : 2

LENGTH : 208

TYPE : amino acid

MOLECULAR TYPE : peptide

FEATURE : HMG-2

ORIGINAL SOURCE : Human

SEQUENCE

Gly Lys Gly Asp Pro Asn Lys Pro Arg Gly Lys Met Ser Ser Tyr Ala Phe Phe
5 10 15

Val Gln Thr Cys Arg Glu Glu His Lys Lys Lys His Pro Asp Ser Ser Val Asn
20 25 30 35

Phe Ala Glu Phe Ser Lys Lys Cys Ser Glu Arg Trp Lys Thr Met Ser Ala Lys
40 45 50

Glu Lys Ser Lys Phe Glu Asp Met Ala Lys Ser Asp Lys Ala Arg Tyr Asp Arg
55 60 65 70

Glu Met Lys Asn Tyr Val Pro Pro Lys Gly Asp Lys Lys Gly Lys Lys Lys Asp
75 80 85 90

Pro Asn Ala Pro Lys Arg Pro Pro Ser Ala Phe Phe Leu Phe Cys Ser Glu His
95 100 105

Arg Pro Lys Ile Lys Ser Glu His Pro Gly Leu Ser Ile Gly Asp Thr Ala Lys
110 115 120 125

Lys Leu Gly Glu Met Trp Ser Glu Gln Ser Ala Lys Asp Lys Gln Pro Tyr Glu
130 135 140

Gln Lys Ala Ala Lys Leu Lys Glu Lys Tyr Glu Lys Asp Ile Ala Ala Tyr Arg
145 150 155 160

Ala Lys Gly Lys Ser Glu Ala Gly Lys Lys Gly Pro Gly Arg Pro Thr Gly Ser
165 170 175 180

Lys Lys Lys Asn Glu Pro Glu Asp Glu Glu Glu Glu Glu Glu Asp Glu
185 190 195

Asp Glu Glu Glu Glu Asp Glu Asp Glu Glu
200 205

[0065]

SEQ ID NO : 3

LENGTH : 214

TYPE : amino acid

MOLECULAR TYPE : peptide

FEATURE : HMG-1

ORIGINAL SOURCE : Bovine

Gly Lys Gly Asp Pro Lys Pro Arg Gly Lys Met Ser Ser Tyr Ala Phe Phe

5 10 15

Val Gln Thr Cys Arg Glu Glu His Lys Lys Lys His Pro Asp Ala Ser Val Asn

20 25 30 35

Phe Ser Glu Phe Ser Lys Lys Cys Ser Glu Arg Trp Lys Thr Met Ser Ala Lys

40 45 50

Glu Lys Gly Lys Phe Glu Asp Met Ala Lys Ala Asp Lys Ala Arg Tyr Glu Arg

55 60 65 70

Glu Met Lys Thr Tyr Ile Pro Pro Lys Gly Glu Thr Lys Lys Phe Lys Asp

75 80 85 90

Pro Asn Ala Pro Lys Arg Pro Pro Ser Ala Phe Phe Leu Phe Cys Ser Glu Tyr

95 100 105

Arg Pro Lys Ile Lys Gly Glu His Pro Gly Leu Ser Ile Gly Asp Val Ala Lys

110 115 120 125

Lys Leu Gly Glu Met Trp Asn Asn Thr Ala Ala Asp Asp Lys Gln Pro Tyr Glu

130 135 140

Lys Lys Ala Ala Lys Leu Lys Glu Lys Tyr Glu Lys Asp Ile Ala Ala Tyr Arg

145 150 155 160

Ala Lys Gly Lys Pro Asp Ala Ala Lys Lys Gly Val Val Lys Ala Glu Lys Ser

165 170 175 180

Lys Lys Lys Glu Glu Glu Asp Glu Glu Asp Glu Glu Asp Glu Glu Glu

185 190 195

Glu Glu Asp Glu Glu Asp Glu Glu Glu Glu Asp Asp Asp Asp Glu

200 205 210

- 35 -

[0066]

SEQ ID NO : 4

LENGTH : 214

TYPE : amino acid

MOLECULAR TYPE : peptide

FEATURE : HMG-1

ORIGINAL SOURCE : Porcine

SEQUENCE

| | | | | | | | | | | | | | | | | | | |
|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|--|
| Gly | Lys | Gly | Asp | Pro | Lys | Lys | Pro | Arg | Gly | Lys | Met | Ser | Ser | Tyr | Ala | Phe | Phe | |
| | | | | | | | | | | | | | | | | | | |
| | | | | 5 | | | | | 10 | | | | | | 15 | | | |
| Val | Gln | Thr | Cys | Arg | Glu | Glu | His | Lys | Lys | Lys | His | Pro | Asp | Ala | Ser | Val | Asn | |
| | | | | | | | | | | | | | | | | | | |
| | | | | 20 | | | | 25 | | | | 30 | | | | 35 | | |
| Phe | Ser | Glu | Phe | Ser | Lys | Lys | Cys | Ser | Glu | Arg | Trp | Lys | Thr | Met | Ser | Ala | Lys | |
| | | | | | | | | | | | | | | | | | | |
| | | | | 40 | | | | 45 | | | | 50 | | | | | | |
| Glu | Lys | Gly | Lys | Phe | Glu | Asp | Met | Ala | Lys | Ala | Asp | Lys | Ala | Arg | Tyr | Glu | Arg | |
| | | | | | | | | | | | | | | | | | | |
| | | | | 55 | | | 60 | | | 65 | | | | 70 | | | | |
| Glu | Met | Lys | Thr | Tyr | Ile | Pro | Pro | Lys | Gly | Glu | Thr | Lys | Lys | Phe | Lys | Asp | | |
| | | | | | | | | | | | | | | | | | | |
| | | | | 75 | | | 80 | | | 85 | | | | 90 | | | | |
| Pro | Asn | Ala | Pro | Lys | Arg | Pro | Pro | Ser | Ala | Phe | Phe | Leu | Phe | Cys | Ser | Glu | Tyr | |
| | | | | | | | | | | | | | | | | | | |
| | | | | 95 | | | | 100 | | | | 105 | | | | | | |
| Arg | Pro | Lys | Ile | Lys | Gly | Glu | His | Pro | Gly | Leu | Ser | Ile | Gly | Asp | Val | Ala | Lys | |
| | | | | | | | | | | | | | | | | | | |
| | | | | 110 | | | 115 | | | 120 | | | 125 | | | | | |
| Lys | Leu | Gly | Glu | Met | Trp | Asn | Asn | Thr | Ala | Ala | Asp | Asp | Lys | His | Pro | Tyr | Glu | |
| | | | | | | | | | | | | | | | | | | |
| | | | | 130 | | | 135 | | | 140 | | | | | | | | |
| Lys | Lys | Ala | Ala | Lys | Leu | Lys | Glu | Lys | Tyr | Glu | Lys | Asp | Ile | Ala | Ala | Tyr | Arg | |
| | | | | | | | | | | | | | | | | | | |
| | | | | 145 | | | 150 | | | 155 | | | 160 | | | | | |
| Ala | Lys | Gly | Lys | Pro | Asp | Ala | Ala | Lys | Lys | Gly | Val | Val | Lys | Ala | Glu | Lys | Ser | |
| | | | | | | | | | | | | | | | | | | |
| | | | | 165 | | | 170 | | | 175 | | | 180 | | | | | |
| Lys | Lys | Lys | Lys | Glu | Glu | Glu | Glu | Asp | Glu | Glu | Asp | Glu | Glu | Glu | Glu | Glu | Glu | |
| | | | | | | | | | | | | | | | | | | |
| | | | | 185 | | | 190 | | | 195 | | | | | | | | |
| Glu | Glu | Asp | Glu | Glu | Asp | Glu | Glu | Glu | Glu | Glu | Asp | Asp | Asp | Asp | Asp | Asp | Glu | |
| | | | | | | | | | | | | | | | | | | |
| | | | | 200 | | | 205 | | | 210 | | | | | | | | |

[0067]

SEQ ID NO : 5

LENGTH : 214

TYPE : amino acid

MOLECULAR TYPE : peptide

FEATURE : HMG-1

ORIGINAL SOURCE : Rat

SEQUENCE

Gly Lys Gly Asp Pro Lys Lys Pro Arg Gly Lys Met Ser Ser Tyr Ala Phe Phe

5 10 15

Val Gln Thr Cys Arg Glu Glu His Lys Lys Lys His Pro Asp Ala Ser Val Asn

20 25 30 35

Phe Ser Glu Phe Ser Lys Lys Cys Ser Glu Arg Trp Lys Thr Met Ser Ala Lys

40 45 50

Glu Lys Gly Lys Phe Glu Asp Met Ala Lys Ala Asp Lys Ala Arg Tyr Glu Arg

55 60 65 70

Glu Met Lys Thr Tyr Ile Pro Pro Lys Gly Glu Thr Lys Lys Phe Lys Asp

75 80 85 90

Pro Asn Ala Pro Lys Arg Pro Pro Ser Ala Phe Phe Leu Phe Cys Ser Glu Tyr

95 100 105

Arg Pro Lys Ile Lys Gly Glu His Pro Gly Leu Ser Ile Gly Asp Val Ala Lys

110 115 120 125

Lys Leu Gly Glu Met Trp Asn Asn Thr Ala Ala Asp Asp Lys His Pro Tyr Glu

130 135 140

Lys Lys Ala Ala Lys Leu Lys Glu Lys Tyr Glu Lys Asp Ile Ala Ala Tyr Arg

145 150 155 160

Ala Lys Gly Lys Pro Asp Ala Ala Lys Lys Gly Val Val Lys Ala Glu Lys Ser

165 170 175 180

Lys Lys Lys Lys Glu Glu Asp Asp Glu Glu Asp Glu Glu Asp Glu Glu

185 190 195

Glu Glu Glu Glu Asp Glu Asp Glu Glu Asp Asp Asp Asp Glu

200 205 210

[0068]

SEQ ID NO : 6

LENGTH : 209

TYPE : amino acid

MOLECULAR TYPE : peptide

FEATURE : HMG-2

ORIGINAL SOURCE : Porcine

SEQUENCE

Gly Lys Gly Asp Pro Asn Lys Pro Arg Gly Lys Met Ser Ser Tyr Ala Phe Phe

5

10

15

Val Gln Thr Cys Arg Glu Glu His Lys Lys His Pro Asp Ser Ser Val Asn

20

25

30

35

Phe Ala Glu Phe Ser Lys Lys Cys Ser Glu Arg Trp Lys Thr Met Ser Ala Lys

40

45

50

Glu Lys Ser Lys Phe Glu Asp Met Ala Lys Ser Asp Lys Ala Arg Tyr Asp Arg

55

60

65

70

Glu Met Lys Asn Tyr Val Pro Pro Lys Gly Asp Lys Lys Gly Lys Lys Lys Asp

75

80

85

90

Pro Asn Ala Pro Lys Arg Pro Pro Ser Ala Phe Phe Leu Phe Cys Ser Glu His

95

100

105

Arg Pro Lys Ile Lys Ser Glu His Pro Gly Leu Ser Ile Gly Asp Thr Ala Lys

110

115

120

125

Lys Leu Gly Glu Met Trp Ser Glu Gln Ser Ala Lys Asp Lys Gln Pro Tyr Glu

130

135

140

Gln Lys Ala Ala Lys Leu Lys Glu Lys Tyr Glu Lys Asp Ile Ala Ala Tyr Arg

145

150

155

160

Ala Lys Gly Lys Gly Glu Ala Gly Lys Lys Gly Pro Gly Arg Pro Thr Gly Ser

165

170

175

180

Lys Lys Lys Asn Glu Pro Glu Asp Glu Glu Glu Glu Glu Glu Glu Asp

185

190

195

Glu Asp Glu Glu Glu Asp Glu Asp Glu Glu

200

205

[0069]

SEQ ID NO : 7

LENGTH : 186

TYPE : amino acid

FEATURE : peptide

FEATURE : partial sequence of HMG-2

ORIGINAL SOURCE : Bovine

SEQUENCE

Gly Lys Gly Asp Pro Asn Lys Pro Arg Gly Lys Met Ser Ser Tyr Ala Phe Phe

5 10 15

Val Gln Thr Ser Arg Glu Glu His Lys Lys Lys His Pro Asp Ala Ser Val Asn

20 25 30 35

Phe Ser Glu/Arg Trp Lys Thr Met Ser Ala Lys Glu Lys Ser Lys Phe Glu Asp

40 45 50

Met Ala Lys Ser Asp Lys Ala Arg Tyr Asp Arg Glu Met Lys Asn Tyr Val Pro

55 60 65 70

Pro Lys Gly Asp Lys Lys Gly Lys Lys Asp Pro Asn Ala Pro Lys Arg Pro

75 80 85 90

Pro Ser Ala Phe Phe Leu Phe Ser Ala Glu His Arg Pro Lys Ile Lys Ala Glu

95 100 105

His Pro Gly Leu Ser Ile Gly Asp Thr Ala Lys Lys Leu Gly Glu Met Trp Ser

110 115 120 125

Gln Gln Ser Ala Lys Asp Lys Gln Pro Tyr Glu Gln Lys Ala Ser Lys Leu Lys

130 135 140

Glu Lys Tyr Glu Lys Xaa Ala Ala Tyr Arg Ala Lys Gly Lys Ser Glu Ala Gly

145 150 155 160

Lys Lys Gly Pro Gly Arg Pro Thr Gly Ser Lys Lys Lys Asn Glu Pro Glu Asp

165 170 175 180

Glu Glu Glu Glu Glu

[0070]

SEQ ID NO : 8

LENGTH : 206

TYPE : amino acid

MOLECULAR TYPE : peptide

FEATURE : HMG-2

ORIGINAL SOURCE : Chiken

SEQUENCE

Gly Lys Gly Asp Pro Asn Lys Pro Arg Gly Lys Met Ser Ser Tyr Ala Tyr Phe

5 10 15

Val Gln Thr Cys Pro Arg Glu His Lys Lys Lys His Pro Asp Ser Ser Val Asn

20 25 30 35

Phe Ala Glu Phe Ser Arg Lys Cys Ser Glu Arg Trp Lys Thr Met Ser Ser Lys

40 45 50

Glu Lys Gly Lys Phe Glu Glu Met Ala Lys Gly Asp Lys Ala Arg Tyr Asp Arg

55 60 65 70

Glu Met Lys Asn Tyr Val Pro Pro Lys Gly Glu Lys Lys Gly Lys Lys Asp

75 80 85 90

Pro Asn Ala Pro Lys Arg Pro Pro Ser Ala Phe Phe Leu Phe Cys Ser Glu His

95 100 105

Arg Pro Lys Ile Lys Asn Asp His Pro Gly Leu Ser Ile Gly Asp Thr Ala Lys

110 115 120 125

Lys Leu Gly Glu Met Trp Ser Glu Gln Ser Ala Lys Asp Lys Gln Pro Tyr Glu

130 135 140

Gln Lys Ala Ala Lys Leu Lys Glu Lys Tyr Glu Lys Asp Ile Ala Ala Tyr Arg

145 150 155 160

Ala Lys Ser Lys Ser Asp Ala Gly Lys Lys Gly Pro Gly Arg Pro Ala Gly Ser

165 170 175 180

Lys Lys Lys Ala Glu Pro Glu Glu Glu Glu Glu Glu Asp Glu Glu Glu

185 190 195

Glu Glu Glu Glu Glu Asp Glu Glu

200 205

- 40 -

[0071]

SEQ ID NO : 9

LENGTH : 201

TYPE : amino acid

MOLECULAR TYPE : peptide

FEATURE : HMG-2a

ORIGINAL SOURCE : Chiken

Ala Lys Gly Asp Pro Lys Lys Pro Lys Gly Lys Met Ser Ala Tyr Ala Phe Phe

5 10 15

Val Gln Thr Cys Arg Glu Glu His Lys Lys Lys Asn Pro Glu Val Pro Val Asn

20 25 30 35

Phe Ala Glu Phe Ser Lys Lys Cys Ser Glu Arg Trp Lys Thr Met Ser Ser Lys

40 45 50

Glu Lys Ala Lys Phe Asp Glu Met Ala Lys Ala Asp Lys Val Arg Tyr Asp Arg

55 60 65 70

Glu Met Lys Asp Tyr Gly Pro Ala Lys Gly Gly Lys Lys Lys Asp Pro Asn

75 80 85 90

Ala Pro Lys Arg Pro Pro Ser Gly Phe Phe Leu Phe Cys Ser Glu Phe Arg Pro

95 100 105

Lys Ile Lys Ser Thr Asn Pro Gly Ile Ser Ile Gly Asp Val Ala Lys Lys Leu

110 115 120 125

Gly Glu Met Trp Asn Asn Leu Ser Asp Gly Glu Lys Gln Pro Tyr Asn Asn Lys

130 135 140

Ala Ala Lys Leu Lys Glu Lys Tyr Glu Lys Asp Val Ala Asp Tyr Lys Ser Lys

145 150 155 160

Gly Lys Phe Asp Gly Ala Lys Gly Ala Ala Thr Lys Ala Ala Arg Lys Lys Val

165 170 175 180

Glu Glu Glu Asp Glu Glu Glu Glu Asp Glu Glu Glu Glu Asp Glu Asp Asp

185 190 195

Asp Asp Glu

200

- 41 -

[0072]

SEQ ID NO : 10

LENGTH : 208

TYPE : amino acid

MOLECULAR TYPE : peptide

ORIGINAL SOURCE : Mouse

FEATURE : HMG-2

Gly Lys Gly Asp Pro Ile Lys Pro Leu Gly Lys Met Ser Ser Tyr Ala Phe Phe

5 10 15

Val Gln Thr Cys Arg Glu Glu His Lys Lys Lys His Pro Asn Ser Ser Val Asn

20 25 30 35

Phe Ala Glu Ile Ser Lys Lys Cys Ser Lys Arg Trp Lys Thr Met Ser Ala Lys

40 45 50

Glu Asn Ser Lys Phe Glu Asp Leu Ala Lys Ser Asp Lys Ala Cys Tyr Tyr Arg

55 60 65 70

Glu Met Lys Asn Tyr Val Ser Pro Lys Gly Asp Lys Lys Gly Lys Lys Lys Asp

75 80 85 90

Pro Asn Ala Pro Lys Arg Pro Pro Ser Ala Phe Cys Leu Phe Cys Ser Glu Asn

95 100 105

Arg Pro Lys Ile Lys Ile Glu Tyr Pro Gly Leu Ser Ile Gly Asp Thr Ala Lys

110 115 120 125

Lys Leu Gly Glu Met Trp Ser Glu Gln Ser Ala Lys Glu Lys Gln Pro Tyr Glu

130 135 140

Gln Lys Ala Ala Lys Leu Lys Glu Lys Tyr Glu Lys Asp Phe Ala Ala Tyr Arg

145 150 155 160

Val Lys Gly Lys Ser Glu Ala Gly Lys Lys Gly Pro Gly Arg Pro Ala Gly Ser

165 170 175 180

Lys Lys Lys Asn Asp Ser Glu Asp Glu Glu Glu Glu Glu Glu Glu Glu

185 190 195

Asp Glu Glu Gly Glu Glu Asp Glu Glu

200 205

- 42 -

[0073]

SEQ ID NO : 11

LENGTH : 32

TYPE : amino acid

MOLECULAR TYPE : peptide

FRAGMENT TYPE : N-terminal fragment of 28KDa

ORIGINAL SOURCE

CELL TYPE : neutrophil cell derived from promyelocytic leukemia

CELL LINE : HL-60 cell line (ATCC CCL-240)

FEATURE

IDENTIFICATION METHOD: E

SEQUENCE

Gly Lys Gly Asp Pro Asn Lys Pro Arg Gly Lys Met Ser Ser Tyr Ala Phe Phe

5

10

15

Val Gln Thr Xaa Arg Glu Glu His Lys Lys Lys His Pro Asp

20

25

30

[0074]

SEQ ID NO : 12

LENGTH : 32

TYPE : amino acid

MOLECULAR TYPE : peptide

FRAGMENT TYPE : N-terminal fragment of 29KDa

ORIGINAL SOURCE

CELL TYPE : neutrophil cell derived from promyelocytic leukemia

CELL LINE : HL-60 cell line (ATCC CCL-240)

FEATURE

IDENTIFICATION METHOD : E

Gly Lys Gly Asp Pro Lys Lys Pro Arg Gly Lys Met Ser Ser Tyr Ala Phe Phe

5

10

15

Val Gln Thr Xaa Arg Glu Glu His Lys Lys Lys His Pro Asp

20

25

30

[Brief Description of the Drawings]

[Figure 1]

A view showing the results of Western blotting performed on neutrophil cytoplasm lysate (antigen), separated from the peripheral blood of healthy persons after electrophoresis on SDS-PAGE using purified IgG (antibody) obtained from the serum of five ulcerative colitis patients and three healthy persons.

[Figure 2]

A view showing the pattern of Western blotting performed using cytoplasm lysates of neutrophil and HL-60 as antigens and also using serum of 28kDa antigen-positive patients as a probe.

[Figure 3]

A view showing a pattern obtained with HPLC, in which the No. 9 peak includes the 28kDa and 29kDa antigens.

[Figure 4]

A view showing a pattern obtained with HPLC, in which

the No. 5 peak includes the 28kDa antigen and the No. 6 peak includes the 29kDa antigen.

[Figure 5]

A view showing a pattern of Western blotting having No. 5 (28kDa) and No. 6 (29kDa) peaks. The bands at the 28kDa and 29kDa are recovered and the amino acid sequence is analyzed.

[Figure 6]

A view showing the results of Western blotting performed on 5 ulcerative colitis patients.

[Figure 7]

A view showing the dose dependent curve obtained by the ELISA method using the serum from the positive patients.

[Figure 8]

A view comparing amino acid sequences of human, porcine, bovine and rat HMG-1.

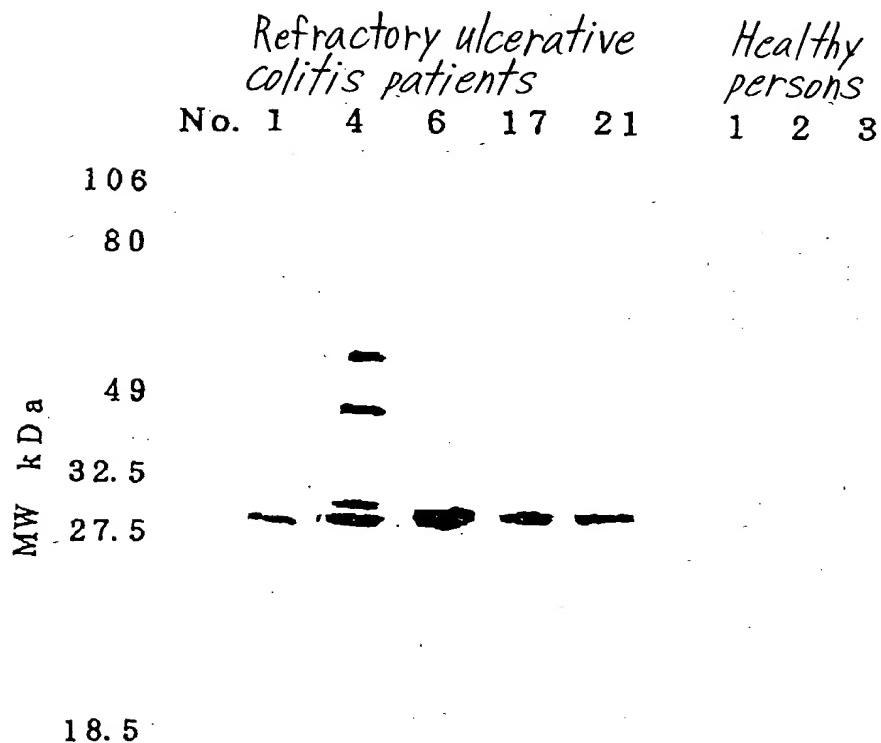
[Figure 9]

A view comparing amino acid sequences of human, porcine, bovine and rat HMG-2.

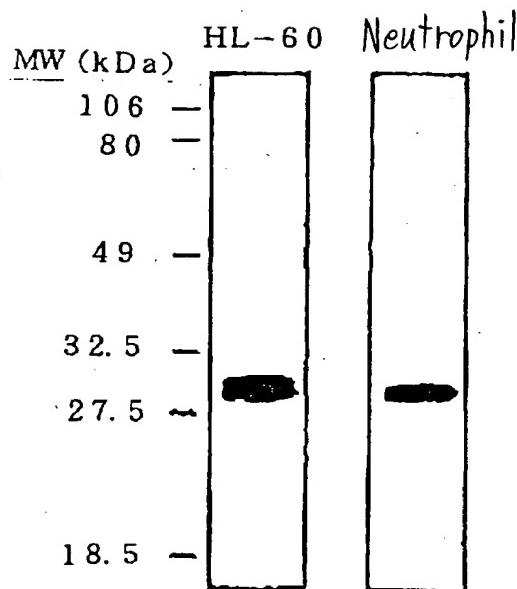
-1-

[Name of the Document] DRAWINGS

[Fig. 1]

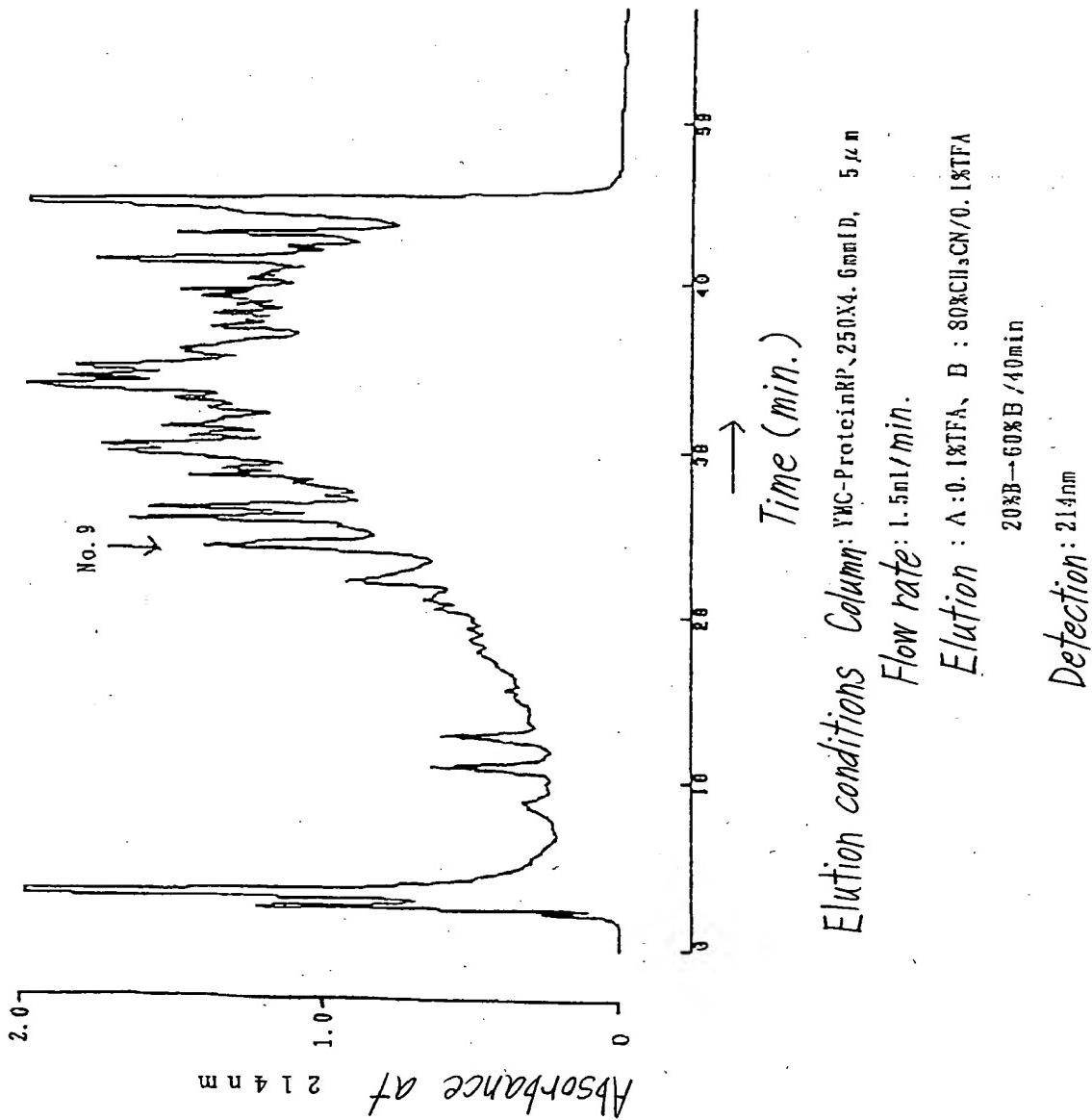


[Fig. 2]



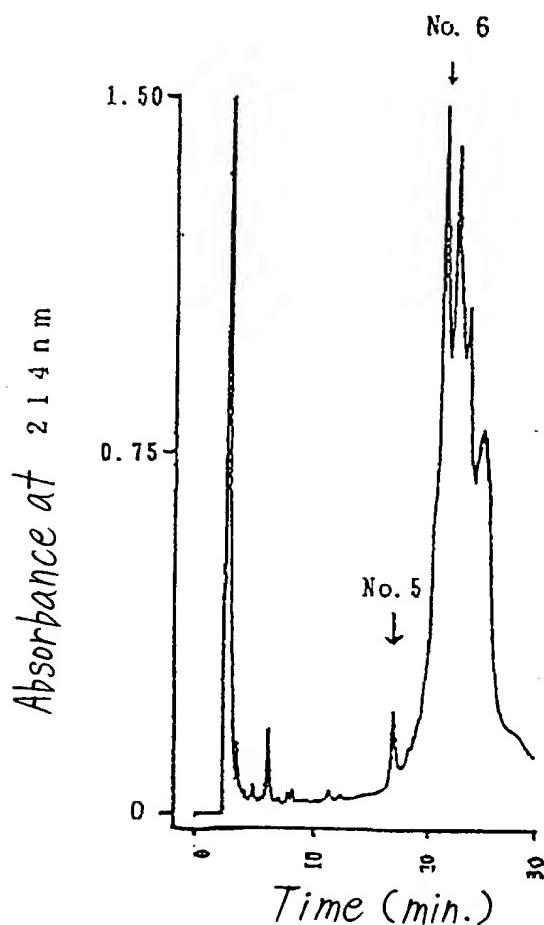
-2-

[Fig. 3]



- 3 -

[Fig. 4]



Elution conditions Column: YMC-ProteinRP, 250X4.6mmID. 5 μ m

Flow rate : 1.5ml/min.

Elution : A : 0. 1%TFA, B : 80%CH₃CN/0. 1%TFA
30%B → 45% B /30min

Detection : 214nm

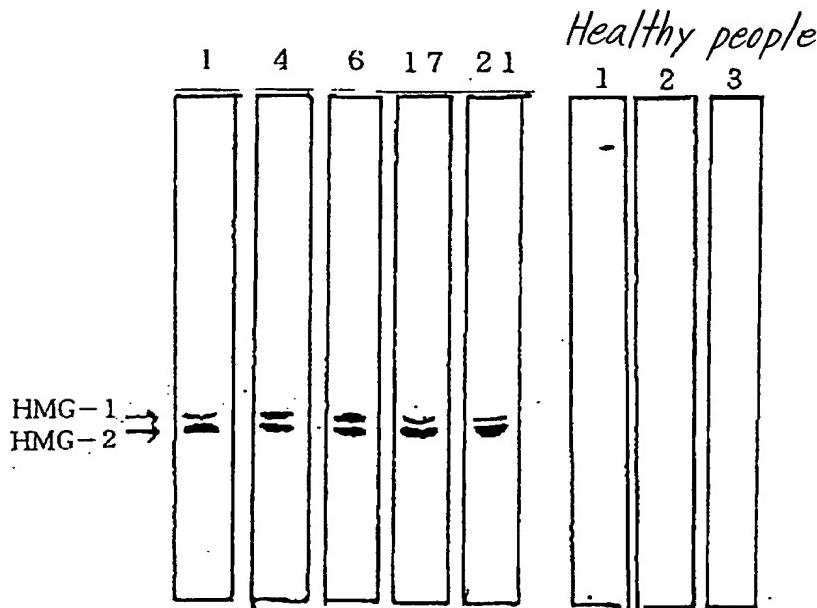
-4-

[Fig. 5]

- 1 2 3
-
1. Positive control
 2. Purified 28kDa antigen
 3. Purified 29kDa antigen

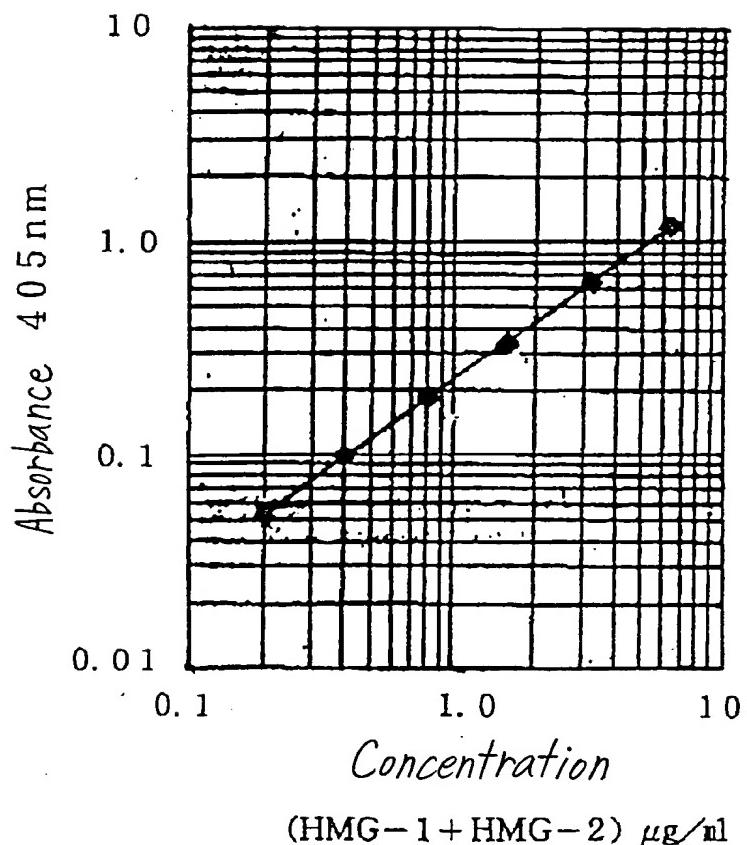
— — — ← 29kDa
 ← 28kDa

[Fig. 6]



-5-

[Fig. 7]



-6-

[Fig. 8]

Comparison among human, porcine, bovine and rat HMG-1
 "I" indicates the same amino acid with that of human HMG-1.

Human 1 GKGDPKPRGK~~I~~S~~I~~YAFFYQTCREEHKKKEPDASVNPFSEFSKKCSE~~R~~KT 50

Porcine 1 GKGDPKPRGK~~I~~S~~I~~YAFFYQTCREEHKKHPDASVNPFSEFSKKCSE~~R~~KT 50

Bovine 1 GKGDPKPRGK~~I~~S~~I~~YAFFYQTCREEHKKHPDASVNPFSEFSKYCSE~~R~~KT 50

Rat 1 GKGDPKPRGK~~I~~S~~I~~YAFFYQTCREEHKKHPDASVNPFSEFSKKCSE~~R~~KT 50

Human 51 I~~S~~A~~K~~E~~G~~KFED~~W~~A~~K~~D~~K~~ARYE~~R~~E~~N~~X~~T~~Y~~I~~PPKG~~E~~T~~KK~~F~~K~~D~~P~~N~~A~~P~~K~~R~~P~~PSA 100

Porcine 51 I~~S~~A~~K~~E~~G~~KFED~~W~~A~~K~~D~~K~~ARYE~~R~~E~~N~~X~~T~~Y~~I~~PPKG~~E~~T~~KK~~F~~K~~D~~P~~N~~A~~P~~K~~R~~P~~PSA 100

Bovine 51 I~~S~~A~~K~~E~~G~~KFED~~W~~A~~K~~D~~K~~ARYE~~R~~E~~N~~X~~T~~Y~~I~~PPKG~~E~~T~~KK~~F~~K~~D~~P~~N~~A~~P~~K~~R~~P~~PSA 100

Rat 51 I~~S~~A~~K~~E~~G~~KFED~~W~~A~~K~~D~~K~~ARYE~~R~~E~~N~~X~~T~~Y~~I~~PPKG~~E~~T~~KK~~F~~K~~D~~P~~N~~A~~P~~K~~R~~P~~PSA 100

Human 101 FFLFCSEYRP~~I~~KGEHPGLS~~I~~G~~D~~V~~A~~K~~E~~L~~G~~E~~W~~NNTAADDKQPYEKKA~~A~~~~K~~L 150

Porcine 101 FFLFCSEYRP~~I~~KGEHPGLS~~I~~G~~D~~V~~A~~K~~E~~L~~G~~E~~W~~NNTAADDKHPYEKKA~~A~~~~K~~L 150

Bovine 101 FFLFCSEYRP~~I~~KGEHPGLS~~I~~G~~D~~V~~A~~K~~E~~L~~G~~E~~W~~NNTAADDKQPYEKKA~~A~~~~K~~L 150

Rat 101 FFLFCSEYRP~~I~~KGEHPGLS~~I~~G~~D~~V~~A~~K~~E~~L~~G~~E~~W~~NNTAADDKQPYEKKA~~A~~~~K~~L 150

Human 151 KEKYEKDIAAYRAK~~K~~KPDA~~A~~EKG~~V~~VKA~~E~~K~~S~~KKKEEEEEEDEEDEEEEEE 200

Porcine 151 KEKYEKDIAAYRAK~~K~~KPDA~~A~~K~~G~~CVVKA~~E~~K~~S~~KKKEEEEEEDEEDEEEEEE 200

Bovine 151 KEKYEKDIAAYRAK~~C~~KPDA~~A~~K~~G~~CVVKA~~E~~K~~S~~KKKEEEEEEDEEDEEEEEE 200

Rat 151 KEKYEKDIAAYRAK~~G~~KPDA~~A~~KK~~G~~VVKA~~E~~K~~S~~KKKEEEEEEDEEDEEEEEE 200

Human 201 DEEDEDEEEEDDDDE 214

||||| |||||

Porcine 201 DEEDEEEEDDDDE 214

||||| |||||

Bovine 201 DEEDEEEEDDDDE 214

||||| |||||

Rat 201 EEEDEDEEEEDDDDE 214

[Fig. 9]

*Comparison among human, porcine, bovine and rat HMG-2
"I" indicates the same amino acid with that of human HMG-2.*

| | |
|---------|--|
| Human | 1 GKGDPNPKPRGKMSYYAFFYQTCTREEHKKHPDSSYNFAEFSKKCSEVKT 50 |
| Porcine | 1 GKGDPNPKPRGKMSYYAFFYQTCTREEHKKHPDSSYNFAEFSKKCSEVKT 50 |
| Bovine | 1 GKGDPNPKPRGKMSYYAFFYQTSREEHKKHPDASVNF---S---ERVKT 50 |
| Rat | 1 CKCDPIKPLGKMSYYAFFYQTCTREEHKKBPNSSVNFAEISKKCSKRVKT 50 |
| Human | 51 WSAKEKSEFEDWAKSDKARYDREMKNYYPPKGDKKKDPNAPKRPPSA 100 |
| Porcine | 51 WSAKEKSKFEDWAKSDKARYDREMKNYYPPKGDKKKDPNAPKRPPSA 100 |
| Bovine | 51 WSAKEKSXKFEDWAKSDKARYDREMKNYYPPKGDKKKDPNAPKRPPSA 100 |
| Rat | 51 WSAKENSKFEDLAKSDKACYYREKHNYSPLGDKKKDPNAPKRPPSA 100 |
| Human | 101 FFLFCSEHRPKIKSEHPGLSIGDTAKKLGEVSEQSAKDKQPYEQKAAL 150 |
| Porcine | 101 FFLFCSEHRPKIKSEHPGLSIGDTAKKLGEVSEQSAKDKQPYEQKAAL 150 |
| Bovine | 101 FFLFSAEHRPKIAEHPGLSIGDTAKKLGEVSQQSAKDKQPYEQKASKL 150 |
| Rat | 101 FCLFCSENRPKIKIEYPGLSIGDTAKKLGEVSEQSAKEKQPYEQKAAL 150 |
| Human | 151 KEKYEKDIAAYRAKGKSEAGKKGPGRPTGSKKNEPEDEEEEEEE-DED 199 |
| Porcine | 151 KEKYEKDIAAYRAKGKSEAGKKGPGRPTGSKKNEPEDEEEEEEEDED 200 |
| Bovine | 151 KEKYEKXX-AAYRAKGKSEAGKKGPGRPTGSKKNEPEDEEEEE.... 200 |
| Rat | 151 KEKYEKDFAAYRVKGKSEAGKKGPGRPACSKKNDSEDEEEEEEE-EED 199 |
| Human | 200 EEEEEEDEE 208 |
| Porcine | 201 EEEEEEDEE 209 |
| Bovine | 201 208 |
| Rat | 201 EEEEEEDEE 208 |

[Name of the Document] ABSTRACT

[Abstract]

[Problems] To specifically detect autoimmune disease and/or inflammatory disease patients.

[Means for Solve the Problems] A diagnostic drug and a diagnostic kit for autoimmune diseases and/or inflammatory diseases, comprising at least one of a polypeptide selected from an HMG-1 family, a polypeptide selected from an HMG-2 family, a fragment thereof which is reactable with an antibody of autoimmune disease and/or inflammatory disease patients. An antibody of an autoimmune disease patient can be detected using such a drug or kit.

[Selected Figure] None